

Lymphocyte subset proliferative responses of *Mycobacterium bovis*-infected cattle to purified protein derivative

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Abstract

Despite highly successful eradication efforts in several countries, *Mycobacterium bovis* infection of cattle remains a significant health concern worldwide. Immune mechanisms of resistance to and/or clearance of *M. bovis* infection of cattle, however, are unclear. Recent studies have provided evidence supporting a role for CD4⁺, CD8⁺, and $\gamma\delta$ TCR⁺ T cells in the response of cattle to *M. bovis*. In the present study, we utilized a flow cytometric-based proliferation assay to determine the relative contribution of individual lymphocyte subsets in the response to *M. bovis* infection and/or sensitization with mycobacterial purified protein derivative (PPD). Peripheral blood mononuclear cells (PBMC) from *M. bovis*-infected cattle proliferated in response to in vitro stimulation with *M. bovis* PPD. CD4⁺ T cells and $\gamma\delta$ TCR⁺ cells were the predominate subsets of lymphocytes responding to PPD. $\gamma\delta$ TCR⁺ cells also proliferated in non-stimulated cultures; however, the $\gamma\delta$ TCR⁺ cell proliferative response of infected cattle was significantly ($p < 0.05$) greater in PPD-stimulated cultures as compared to non-stimulated cultures. Intradermal injection of PPD for comparative cervical testing (CCT) induced a boost in the in vitro proliferative response of CD4⁺ but not $\gamma\delta$ TCR⁺ cells of infected cattle. Administration of PPD for CCT also boosted interferon- γ (IFN- γ) production by PBMC of infected cattle following in vitro stimulation with

Abbreviations: BCG, bacillus Calmette-Guerin; CCT, comparative cervical test; cfu, colony forming units; FBS, fetal bovine sera; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PPDa, purified protein derivative — *M. avium*; PPDb, purified protein derivative — *M. bovis*; PPD, purified protein derivative; SI, stimulation indices

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M. bovis PPD. Injection of PPD for CCT did not, however, elicit a proliferative or IFN- γ response in cells isolated from non-infected cattle. These data indicate that CD4⁺ and $\gamma\delta$ TCR⁺ cells of *M. bovis*-infected cattle proliferate in a recall response to *M. bovis* PPD and that the CD4⁺ cell response is boosted by intradermal injection with PPD for CCT. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Mycobacterium bovis infection remains a significant health problem worldwide for cattle and other animal species (Neill et al., 1994). Although nearly eradicated from most developed countries, wildlife reservoirs such as badgers (*Meles meles*) and possums (*Trichosurus vulpecula*) have hindered eradication efforts (Barrow and Gallagher, 1981; Coleman, 1988). Vaccination, including the use of bacillus Calmette-Guerin (BCG), has been examined as an option to eradication measures (Buddle et al., 1995; Newell and Hewinson, 1995). As with human tuberculosis, the efficacy of BCG in cattle is variable and may interfere with interpretation of intradermal skin testing (Buddle et al., 1995). Thus, there is a renewed interest in the development of improved vaccines for use in cattle, wildlife reservoirs, and/or farmed *Cervidae* for prevention of *M. bovis*. This interest justifies further studies to determine mechanisms of protective immunity to *M. bovis* infection.

T cell-mediated immunity is considered essential for clearance of mycobacterial infections (Orme and Collins, 1983; Pedrazzini et al., 1987). Recently, it was determined that fractionated bovine CD4⁺ or CD8⁺ T cells from *M. bovis*-infected cattle proliferate and produce interferon- γ (IFN- γ) in a recall response to *M. bovis* antigen (Liebana et al., 1999). In addition to CD4⁺ and CD8⁺ T cells, $\gamma\delta$ TCR⁺ cells are also implicated in the response of cattle to *M. bovis* infection (Pollock et al., 1996; Rhodes et al., 2000a,b; Smith et al., 1999). Indeed, $\gamma\delta$ T cells of other species respond to various mycobacterial antigens and accumulate at sites of infection (Augustin et al., 1989; Boom et al., 1992; Janis et al., 1989; Tanaka et al., 1995). Production of IFN- γ by CD4⁺, CD8⁺, and/or $\gamma\delta$ TCR⁺ cells leading to activation of macrophages and killing of intracellular mycobacteria is one plausible mechanism of clearance of *M. bovis* (Carpenter et al., 1997, 1998; Kaufmann, 1997; Liebana et al., 1999; Ng et al., 1997). However, mycobacteria-specific cytotoxic T cells (both CD4⁺ or CD8⁺) have also been described, indicating that cytotoxic function may also be important in the clearance of *M. bovis* (Mutis et al., 1993; Orme et al., 1993; Pithie et al., 1992).

In the present study, we utilized a flow cytometric-based proliferation assay to detect proliferation of lymphocytes from *M. bovis*-infected cattle. This assay affords the capability of distinguishing individual lymphocyte subsets proliferating in peripheral blood mononuclear cell (PBMC) cultures. Effects of infection and in vivo stimulation with purified protein derivative (PPD) on in vitro proliferative and IFN- γ responses are described.

2. Materials and methods

2.1. Experimental design, *M. bovis* challenge procedure

Cattle were exposed to *M. bovis* through indirect contact with experimentally infected white-tailed deer (*Odocoileus virginianus*). Briefly, twelve 11-month old deer were experimentally challenged by instillation of 7×10^5 cfu *M. bovis* (strain 1315 originally isolated from a white-tailed deer in Michigan in 1995) into the crypts of the palatine tonsil (Palmer et al., 1999). After 2 weeks, pens where the deer were housed were disinfected and nine 6-month old cattle were introduced into the barn. Cattle and deer were divided into three groups of three and four animals each, respectively. Each group of four deer was paired with a group of three cattle. After several hours, the deer were moved to a holding pen and the cattle were moved to the pens that had been occupied by the deer (e.g., without prior cleaning of the deer pens and without removal of excess grain and hay fed to the deer). Cattle were, thus, exposed to an environment in which *M. bovis*-infected deer had previously occupied. The cattle pens were cleaned and the deer (in the holding pens) were then moved to the clean pens. This process was repeated daily for 80 days. Post-mortem examinations of cattle were conducted beginning 177 days after they were introduced into the barn.

Cattle exposed to *M. bovis* were tested with the caudal fold test for a delayed hypersensitive response to *M. bovis* PPD (PPDb) using standard methods prior to initiation of the study (USDA, 1999). On day 77 and 174 of the study, *M. bovis*-exposed cattle were repeat skin tested using the comparative cervical test (CCT) as described (USDA, 1999). A group of three 6-month old cattle served as “control-tested” cattle. These animals were not exposed to *M. bovis* but were skin tested at the same three time points as the exposed cattle. Two cattle that were skin tested once at day 174 served as “control” cattle.

2.2. Lymphocyte blastogenesis assay

Mononuclear cells were isolated from buffy coat fractions of peripheral blood collected in 2X-acid citrate dextrose using standard procedures (Burton and Kehrli, 1996). Wells of 96-well flat-bottomed microtiter plates (Falcon, Becton Dickinson; Lincoln Park, NJ) were seeded with 2×10^5 mononuclear cells in a total volume of 200 μ l per well. The medium was RPMI 1640 supplemented with 25 mM HEPES buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol (Sigma), and 10% fetal bovine sera (FBS). The wells contained medium plus PPDb or medium alone (no stimulation). Twofold dilutions from 20 to 1.25 μ g/ml were used for PPD stimulation of PBMC. Cells in wells were then incubated for 5 days at 37°C in 5% CO₂ in air. After 5 days, 0.5 μ Ci of (methyl-[³H] thymidine (specific activity 6.7 Ci mmol⁻¹, Amersham Life Science, Arlington Heights, IL) in 10 μ l of medium was added to each well, and cells incubated for an additional 20 h. The well contents were harvested on to glass fiber filters with a PHD Cell Harvester (Cambridge Technology, Cambridge, MA) and the incorporated radioactivity measured by liquid scintillation counting. Treatments were run in triplicate and stimulation indices (SI) calculated by dividing counts per minute of

stimulated wells by counts per minute from non-stimulated wells. Data are presented as mean SI \pm SEM.

2.3. PKH67 proliferation assay

The PKH67 proliferation assay was performed according to manufacturers instructions (Sigma) and as previously described for PKH2 (Waters et al., 1999a,b). Briefly, 2×10^7 PBMC were centrifuged (400g) for 5 min, supernatants aspirated, and cells resuspended in 1 ml of diluent (Sigma). Cells, in diluent, were added to 1 ml of PKH67 green fluorescent dye (2×10^{-6} M) and incubated 5 min followed by a 1 min incubation with 2 ml of FBS to stop the reaction. Cells were then washed three times with RPMI 1640. PKH67 stained cells were added to quadruplicate wells (2×10^5 per well) of a 96 well round-bottomed microtiter plate in medium (no stimulation) or medium plus either 1 or 10 μ g/ml PPD_b. Cells in wells were then incubated at 37°C in a 5% CO₂ humidified chamber. After 6 days of culture, cells were analyzed by flow cytometry for PKH67 staining as well as cell surface marker expression. Modfit Proliferation Wizard (Verity Software House, Topsham, ME) and CellQuest software (Becton Dickinson, San Jose, CA) were used for cell proliferation and phenotype analyses. Proliferation profiles were determined as the number of cells proliferating in antigen-stimulated wells minus the number of cells proliferating in non-stimulated wells for both gated (i.e., CD3⁺, CD4⁺, CD8⁺, $\gamma\delta$ TCR⁺, or B cells) or ungated (total PBMC) populations. Data are presented as the mean number of cells that had proliferated/10,000 PBMC \pm SEM.

2.4. Preparation and analysis of cells by flow cytometry

Mononuclear cells were analyzed for expression of cell surface antigens by flow cytometry. Cells (2×10^6 /ml) in 100 μ l balanced salt solution with 1% FBS and 0.1% sodium azide (FACS buffer) were stained with 100 μ l of primary antibody to leukocyte surface antigens MM1A, anti-CD3; GC50A1, anti-CD4; BAQ111A, anti-CD8 α ; BAT82A, anti-CD8 β ; GB21A, anti- $\gamma\delta$ TCR; and BAQ155A, anti-B cell (all obtained from VMRD, Pullman, Washington, DC). Following a 15 min incubation, cells were centrifuged (400g) for 2 min and resuspended in 100 μ l of PE-conjugated goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Birmingham, AL) and PerCP-conjugated goat anti-mouse immunoglobulin (Becton Dickinson). Cells were then incubated for an additional 15 min, centrifuged (400g) for 2 min, resuspended in FACS buffer and analyzed using a Becton Dickinson FACScan flow cytometer (5000–10,000 events from each sample, three color analysis).

2.5. Interferon- γ ELISA

Heparinized blood (1.5 ml) was added to 2 ml microcentrifuge tubes containing either 0.1 ml of 300 μ g/ml PPD_b (0.20 μ g/ml final concentration) or 0.1 ml of PBS (no stimulation) and incubated 22 h at 37°C in a humidified chamber with 5% CO₂. After centrifugation, plasma from each tube was harvested and stored at –20°C for later

analysis of IFN- γ by ELISA using a commercially available test kit (BovigamTM, CSL Limited, Parkville, Vic., Australia).

2.6. Statistics

Data were analyzed either by one-way analysis of variance followed by Tukey–Kramer multiple comparisons test or Student's *t*-test. Differences between groups were considered significant if probability values of $p \leq 0.05$ were obtained.

3. Results

3.1. Infection status

To mimic a natural route of exposure, cattle were infected with *M. bovis* by daily exposure to an environment recently (5 min prior) inhabited by *M. bovis*-infected white-tailed deer. Complete results of bacteriologic culture and gross and histopathologic examination studies are included in a companion paper (Whipple et al., 2000). All *M. bovis*-exposed cattle were classified as reactors by CCT at 77 and 174 days post-exposure to *M. bovis*. All cattle exposed to *M. bovis* developed lesions typical of bovine tuberculosis and *M. bovis* was isolated from each animal. All cattle not exposed to *M. bovis* (e.g., control-tested and control) were classified as negative by CCT.

3.2. Lymphocyte proliferation

Detection of antigen-specific proliferation by measurement of reduction in PKH staining is only recently described for cattle (Waters et al., 1999a). Thus, we compared results of this assay to results obtained with a standard [³H] thymidine uptake proliferation assay (Fig. 1). By both assays, significantly ($p < 0.05$) greater proliferative responses were detected for infected cattle as compared to control non-infected cattle. Prior sensitization of control cattle by CCT (e.g., intradermal injection of 100 μ g PPDb and 40 μ g PPDa; control-tested) did not result in a significant proliferative response as detected by either test (Figs. 1 and 2). However, in vivo administration of PPD for CCT (e.g., intradermal injection of 100 μ g PPDb and 40 μ g PPDa) significantly ($p < 0.05$) boosted the in vitro proliferative responses to PPDb of *M. bovis*-infected cattle (Fig. 2).

3.3. Proliferation of lymphocyte subsets

An advantage of the use of PKH fluorescent dyes to determine lymphocyte proliferation is that this assay can be combined with cell surface marker analysis to determine which subset(s) of lymphocytes have proliferated. Freshly isolated cells were stained with PKH67, cultured for a period of 6 days with or without stimulation, harvested, stained with mAbs to cell surface antigens, and analyzed by flow cytometry for PKH67 fluorescence intensity and cell surface marker expression as previously described (Waters et al., 1999a,b). The stability of the dye incorporation into the lipid membrane of

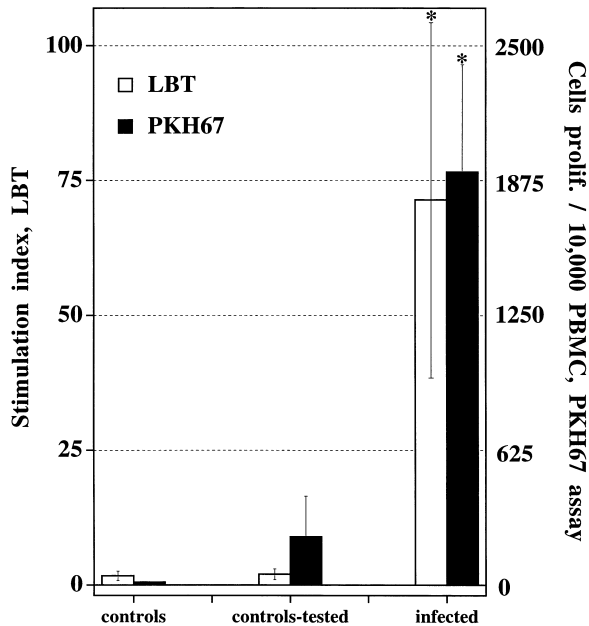


Fig. 1. Comparison of two assays for antigen-specific proliferation of PBMC isolated from control (non-infected, $n = 2$), control-tested (e.g., cattle were tested for *M. bovis* by CCT 2 months prior, $n = 3$), and *M. bovis*-infected cattle ($n = 5$). Open bars represent mean stimulation indices \pm SEM of PBMC in response to 1.25 $\mu\text{g/ml}$ PPD_b as measured by [^3H] thymidine uptake (see Section 3). Closed bars represent the mean number of cells proliferating/10,000 PBMC \pm SEM in response to 1 $\mu\text{g/ml}$ PPD_b as measured by the PKH67 assay (see Section 3). Significantly ($p < 0.05$) greater as compared to controls and control-tested.

the cell ensures that when cells divide the dye is distributed equally between daughter cells. PKH67 staining intensity diminishes with each cell division resulting in a decreased mean fluorescence intensity (Ashley et al., 1993). As shown in Fig. 3, in vitro stimulation of PBMC from *M. bovis*-infected cattle with 10 $\mu\text{g/ml}$ of PPD_b for 6 days results in the generation of numerous lymphoblasts whereas stimulation of PBMC from non-infected cattle with PPD_b does not result in the generation of lymphoblasts. Live cells (e.g., those cells in gate R1) were then analyzed for their cell surface marker expression as well as PKH67 staining intensity. As shown in Table 1, there was a significant ($p < 0.05$) B cell proliferative response to PPD_b by infected cattle as compared to B cell responses from non-infected (e.g., control and control-tested) cattle. However, the overwhelming majority of PBMC from infected cattle responding were $\text{CD}3^+$ cells (significantly greater than responses from non-infected cattle and B cell responses from infected cattle, $p < 0.05$). Of the $\text{CD}3^+$ cells proliferating, the proliferative response of the $\text{CD}4^+$ and $\gamma\delta \text{TCR}^+$ cell populations were significantly ($p < 0.05$) greater than the proliferative response of these respective subsets from control and control-tested cattle whereas the $\text{CD}8^+$ cell response was not significant. The $\gamma\delta \text{TCR}^+$ cell response was significantly ($p < 0.05$) greater than the $\text{CD}4^+$ T cell, $\text{CD}8^+$ T cell, and B cell responses at 155 and 177 days post-exposure.

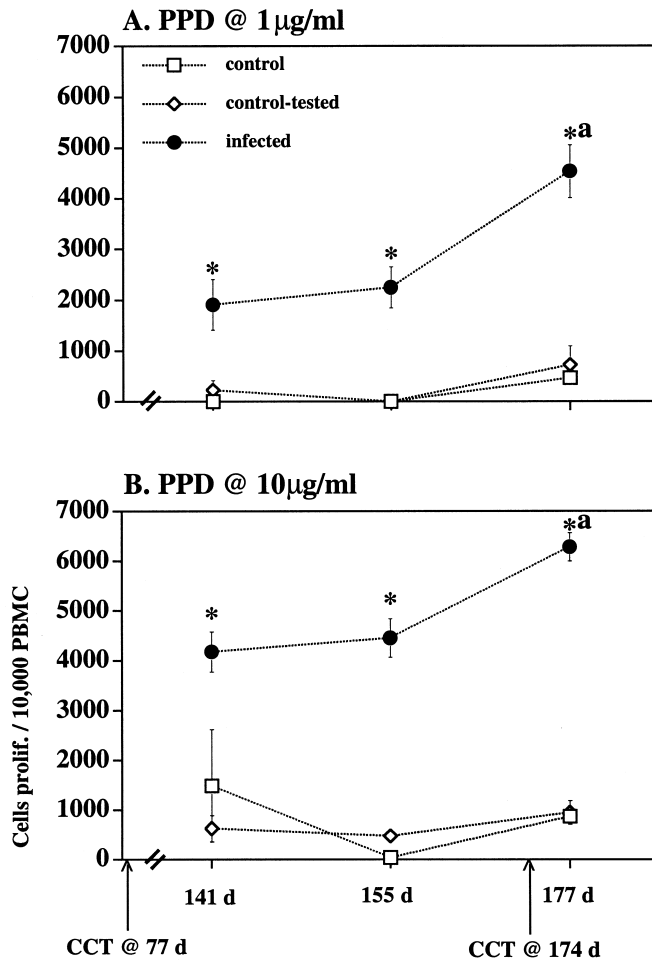


Fig. 2. Temporal dynamics of the proliferative response of control (open squares, $n = 2$), control-tested (open diamonds, $n = 3$), and infected (closed circles, $n = 5$) cattle in response to in vitro stimulation with either 1 µg/ml (A) or 10 µg/ml (B) PPD_b. Mononuclear cells were isolated from the peripheral blood, stained with PKH67, and cultured for 6 days with or without PPD_b stimulation. Values represent the mean number of cells proliferating/10,000 PBMC \pm SEM at the indicated days (x -axis) post-exposure to *M. bovis*. CCT refers to comparative cervical test; control-tested and infected cattle received an intradermal injection of 100 µg PPD_b and 40 µg PPD_a at day 77 of the study, whereas all cattle received 100 µg PPD_b and 40 µg PPD_a at day 174 of the study. Significantly ($p < 0.05$) greater as compared to controls and control-tested. Significantly ($p < 0.05$) greater as compared to mean values from days 141 and 155 for infected cattle.

3.4. CD4⁺ cell proliferation

Proliferative responses of CD4⁺ T cells are considered a hallmark of mycobacterium infection (Orme et al., 1992). As shown in Fig. 4, the CD4⁺ cell proliferative response of *M. bovis*-infected cattle was vigorous. Analysis of PBMC from a representative

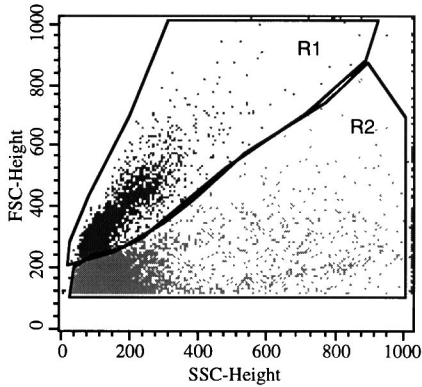
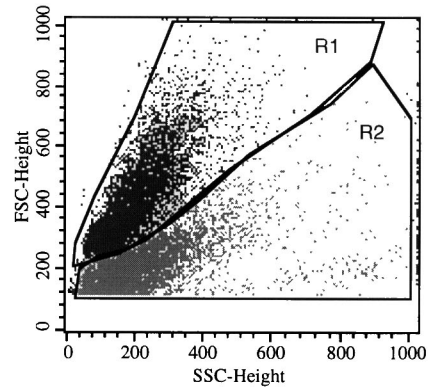
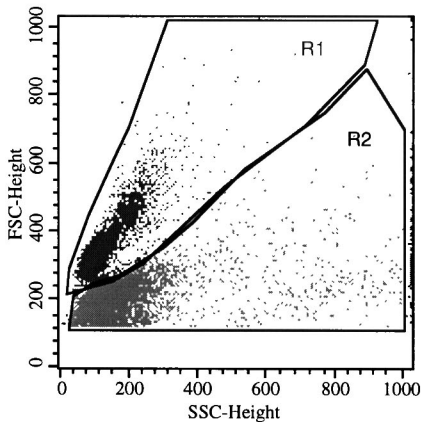
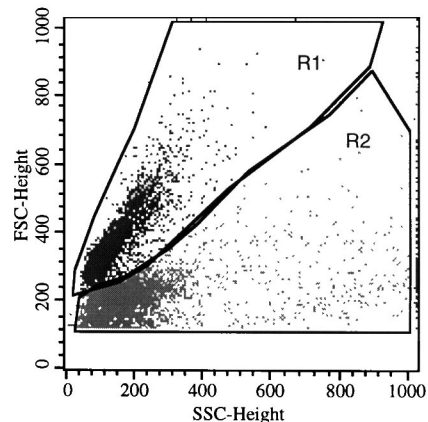
A. Non-stimulated PBMC from an infected animal**B. PPD-stimulated PBMC from an infected animal****C. Non-stimulated PBMC from a non-infected animal****D. PPD-stimulated PBMC from a non-infected animal**

Fig. 3. Scatter plots depicting lymphoblast formation in response to PPD_b stimulation of PBMC from *M. bovis*-infected cattle. Mononuclear cells were isolated from the peripheral blood of a *M. bovis*-infected animal (A and B) and a non-infected animal (C and D). Cells were cultured for 6 days in media alone (A and C) or media plus 10 μ g/ml PPD_b (B and D) and analyzed for forward (y-axis) and side (x-axis) scatter. Note the presence of lymphoblasts (cells with an \uparrow in forward and side light scatter) in the gate designated R1 in B; thus, indicating proliferation. Gate R2 depicts apoptotic, dead, or dying cells (as previously determined, unpublished data). These plots are representative of PBMC from cattle from each of the respective groups.

M. bovis-infected animal demonstrates that 5% of the non-stimulated CD4⁺ cells are PKH67 dim (Fig. 4A), whereas 69% of the PPD_b-stimulated CD4⁺ cells are dim (Fig. 4B). Again, with cell proliferation, PKH67 staining intensity diminishes; thus, PKH67 dim cells have proliferated. Stimulation of PBMC from a representative

Table 1

Proliferation of lymphocyte subsets in response to stimulation with *M. bovis* PPD

	Total PBMC	CD3 ⁺	CD4 ⁺	CD8 ⁺	$\gamma\delta$ TCR ⁺	B Cells
(A) Responses 141 days post-exposure to <i>M. bovis</i> ^a						
Control	1486±1134	1063±595	214±16	162±97	482±192	370±184
Control-tested	627±261	214±38	160±51	112±73	180±93	251±135
Infected	4178±403 ^b	3552±378 ^b	1315±175 ^b	356±129	1534±213 ^{b,c}	842±103 ^d
(B) Responses 155 days post-exposure to <i>M. bovis</i> ^a						
Control	38±38	0±0	0±0	0±0	132±132	0±0
Control-tested	477±67	690±26	162±65	99±58	398±142	89±44
Infected	4453±384 ^b	3869±424 ^b	1080±138 ^b	260±55	2497±437 ^{b,c}	488±32 ^b
(C) Responses 177 days post-exposure to <i>M. bovis</i> , 3 days post-CCT for tested and infected cattle ^a						
Control	872±31	572±382	41±20	4±4	256±157	68±68
Control-tested	952±242	889±211	146±36	86±71	663±355	140±57
Infected	6284±284 ^{b,f}	5567±278 ^{b,f}	1620±153 ^{b,g}	308±68	2464±208 ^{b,c}	653±90 ^b

^a Data represent the mean number of cells that had proliferated per 10,000 PBMC ± SEM in response to stimulation with 10 µg/ml PPD_b. Treatment groups included control cattle (e.g., no exposure to *M. bovis* yet tested by CCT on day 174 of the study for exposure to *M. bovis*, *n* = 2), control-tested cattle (e.g., no exposure to *M. bovis*, yet tested by caudal fold test prior to study and CCT at days 77 and 174 of the study for exposure to *M. bovis*, *n* = 3), and infected cattle (e.g., cattle exposed to *M. bovis* and tested by caudal fold test prior to the study and CCT on days 77 and 174 of the study for exposure to *M. bovis*, *n* = 5). Control-tested and infected cattle received an intradermal injection of 100 µg PPD_b for the caudal fold test prior to the study and 100 µg PPD_b and 40 µg PPD_a for CCT at day 77 and 174 of the study. All cattle received 100 µg PPD_b and 40 µg PPD_a for CCT at day 174 of the study.

^b Significantly (*p* < 0.05) greater than data representing results from control and control-tested cattle for an individual time point and subset (e.g., vertical comparisons within given sample time point).

^c Significantly (*p* < 0.05) greater than data representing CD8⁺ cell and B cell responses from infected cattle (e.g., horizontal comparisons within given sample time point).

^d Significantly (*p* < 0.05) greater than data representing results from control-tested cattle for an individual time point and subset (e.g., vertical comparisons within given sample time point).

^e Significantly (*p* < 0.05) greater than data representing CD4⁺ cell, CD8⁺ cell, and B cell responses from infected cattle (e.g., horizontal comparisons within given sample time point).

^f Significantly (*p* < 0.05) greater than data representing CD3⁺ cell responses from 141 and 155 days post-exposure to *M. bovis*.

^g Significantly (*p* < 0.05) greater than data representing CD4⁺ cell responses from 155 days post-exposure to *M. bovis*.

non-infected animal with PPD_b did not, however, result in diminishment of PKH67 intensity (e.g., 6% of PPD_b-stimulated CD4⁺ cells are dim, Fig. 4D) when compared to non-stimulated cells (e.g., 7% of non-stimulated CD4⁺ cells are dim, Fig. 4C) from the same animal. Thus, the CD4⁺ cell proliferative response to PPD_b occurs only with cells isolated from *M. bovis*-infected cattle.

3.5. $\gamma\delta$ TCR⁺ cell proliferation

The proliferative response of $\gamma\delta$ TCR⁺ cells from *M. bovis*-infected cattle to PPD_b was remarkable in two aspects. As stated above, of the CD3⁺ cells proliferating, the $\gamma\delta$ TCR⁺ cell response was significantly greater than responses from other T cell subsets at 155 and

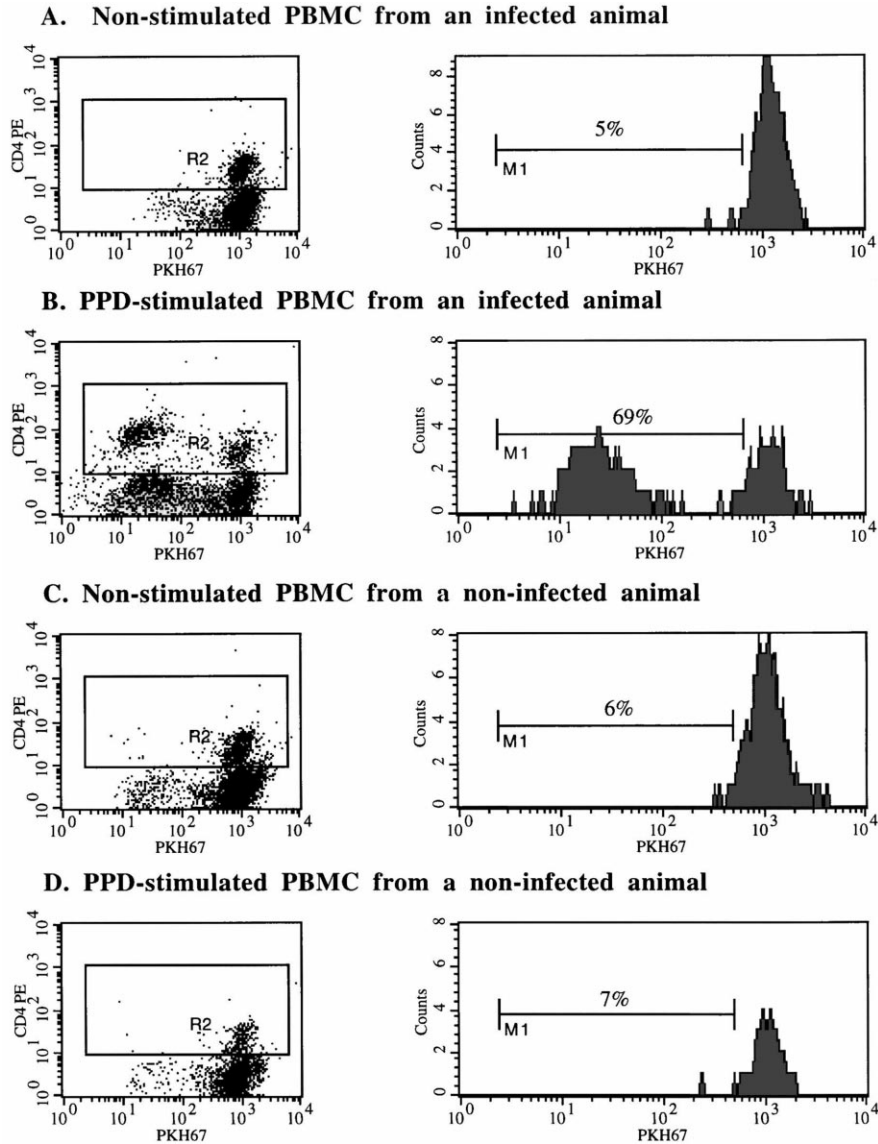


Fig. 4. $CD4^+$ cell proliferation as measured by analysis of PKH67 staining intensity. Histograms on right represent PKH67 analysis of cells included in the R2 gate of scatter plots on the left. Thus, gates are set on $CD4^+$ cells and the percent of cells that are PKH67 dim (e.g., proliferating) are determined (e.g., percent of cells designated in region M1 on histograms on the right). The percentage of PKH67 dim $CD4^+$ cells from non-stimulated PBMC cultures from an infected animal is 5% (A) whereas the percentage of PKH67 dim $CD4^+$ cells from PPD-stimulated cultures from an infected animal is 69% (B). However, PPD stimulation of PBMC from a non-infected animal does not result in a notable increase in PKH67 dim $CD4^+$ cells (7%, D) in comparison to PKH67 dim $CD4^+$ cells in non-stimulated cultures (6%, C). Although results from a single animal per group are demonstrated, similar results were obtained with other animals. These results were obtained from PBMC isolated from animals 141 days post-exposure to *M. bovis*.

177 days post-exposure to *M. bovis* (Table 1). Secondly, $\gamma\delta$ TCR⁺ cell proliferation also accounted for the majority of the background proliferation detected in non-stimulated cultures from both *M. bovis*-infected and non-infected cattle (Fig. 5A and C). However, in PPDb-stimulated cultures from infected cattle, a substantial increase in proliferation was detected in comparison to non-stimulated cultures (Fig. 5B vs. 5A), whereas no increase in proliferation in PPDb-stimulated cultures as compared to non-stimulated cultures was detected from $\gamma\delta$ TCR⁺ cells from non-infected cattle (Fig. 5D vs. 5C). For the determination of proliferation values in Table 1, background proliferation (e.g., proliferation in non-stimulated cultures) was subtracted from the proliferation in PPDb-stimulated cultures of individual subsets. Thus, although $\gamma\delta$ TCR⁺ cells were responsible for the majority of the non-specific proliferation in non-stimulated cultures, a significant ($p < 0.05$) PPDb-specific response was also detected for this subset of T cells (Table 1 and Fig. 5).

3.6. Effect of CCT on lymphocyte subset proliferation

As stated previously, in vivo administration of PPD for CCT results in a boost in the proliferative response to PPDb of cattle infected with *M. bovis* (Fig. 2). Using the PKH67 assay, we next assessed the relative contribution of individual lymphocyte subsets to this boosted response. Three days prior to the last sampling point at 177 days post-exposure to *M. bovis* (e.g., Table 1C), infected, control, and control-tested cattle received an intradermal injection of 100 μ g PPDb and 40 μ g PPDa for CCT. As shown in Table 1, examination of proliferative responses of *M. bovis*-infected cattle demonstrated that there was a significant ($p < 0.05$) increase in proliferating CD3⁺ cells at 177 days post-exposure to *M. bovis* in comparison to responses at 141 and 155 days post-exposure. These results indicate that intradermal injection of PPD boosted the proliferative response of T cells from *M. bovis*-infected cattle. Of the CD3⁺ cells, there was a significant increase in proliferating CD4⁺ cells at 177 days post-exposure in comparison to 155 days post-exposure. The CD4⁺ cell proliferative response had actually diminished from 141 days post-exposure (1315 ± 175) to 155 days post-exposure (1080 ± 138); indicating that the boost of the proliferative response after PPD administration 3 days prior to sampling at 177 days post-exposure (1620 ± 153) is of greater relevance.

3.7. Effect of CCT on IFN- γ responses

Antigen-specific IFN- γ production was measured by analysis of plasma samples obtained from non-stimulated and PPDb-stimulated whole blood samples using a commercially available ELISA kit (CSL). Non-infected cattle, including those cattle that were tested for *M. bovis* by CCT, had mean optical density readings of <0.2 throughout the study. *M. bovis*-infected cattle had mean OD values that steadily increased from 0.3 at 28 days post-exposure to *M. bovis* to 3.7 at 121 days post-exposure. Mean OD values for IFN- γ of infected cattle remained high (>3.0) throughout the remainder of the study. Additionally, as shown in Table 2, administration of PPD for CCT significantly ($p < 0.05$) boosted the IFN- γ response by PBMC of infected cattle. This boost was

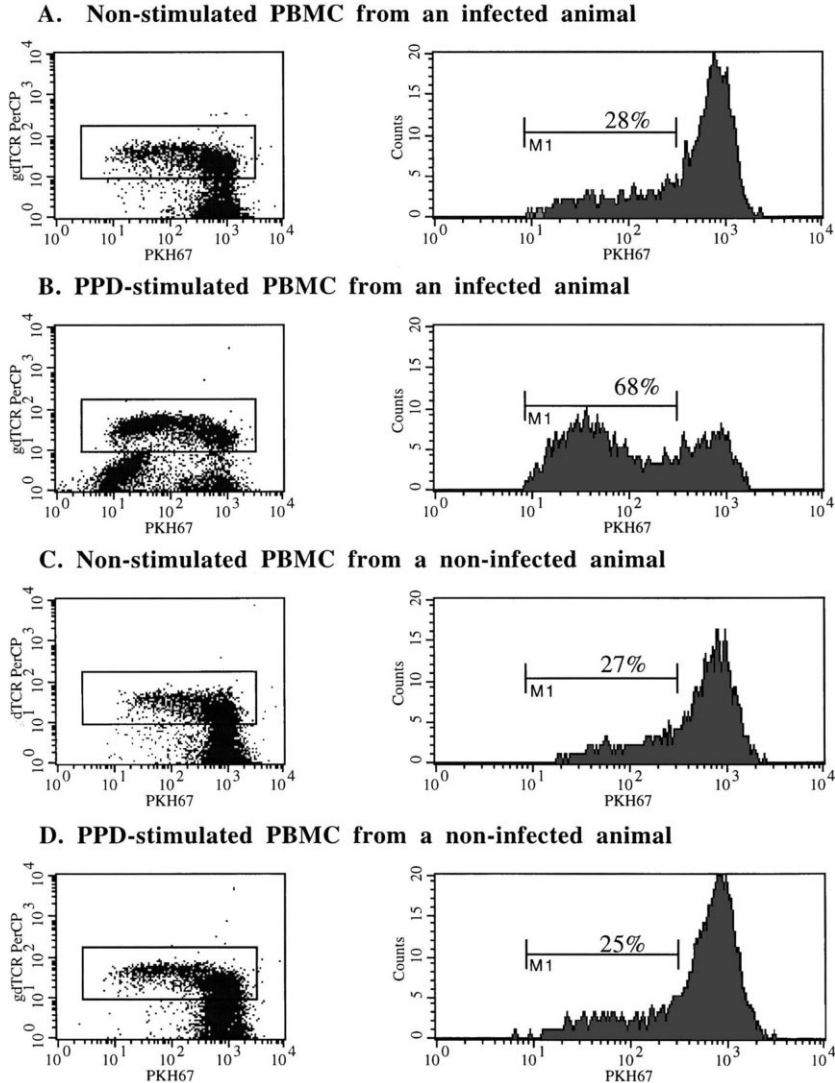


Fig. 5. Non-specific and PPD_b-specific proliferation of $\gamma\delta$ TCR⁺ cells. Histograms on right represent PKH67 analysis of cells included in the R2 gate of scatter plots on the left. Thus, gates are set on $\gamma\delta$ TCR⁺ cells and the percent of cells that are PKH67 dim (e.g., proliferating) are determined (e.g., percent of cells designated in region M1 on histograms on the right). The percentage of PKH67 dim $\gamma\delta$ TCR⁺ cells from non-stimulated PBMC cultures from an infected animal is 28% (A) whereas the percentage of PKH67 dim $\gamma\delta$ TCR⁺ cells from PPD_b-stimulated cultures from an infected animal is 68% (B). However, PPD_b stimulation of PBMC from a non-infected animal does not result in an increase in PKH67 dim $\gamma\delta$ TCR⁺ cells (27%, D) in comparison to PKH67 dim $\gamma\delta$ TCR⁺ cells in non-stimulated cultures (25%, C). Note the large number of PKH67 dim $\gamma\delta$ TCR⁺ cells in non-stimulated cultures from either infected (A) or non-infected cattle (B); indicating non-specific proliferation of this subset. Although results from a single animal per group are demonstrated, similar results were obtained with other animals. These results were obtained from PBMC isolated from animals 177 days post-exposure to *M. bovis*.

Table 2

Administration of PPD for CCT boosts *M. bovis*-specific IFN- γ responses of infected cattle^a

Days post-exposure to <i>M. bovis</i>	77 (day of first PPD administration)	80 (3 days post-first PPD administration)	174 (day of second PPD administration)	177 (3 days post-second PPD administration)
IFN- γ (mean \pm SEM)	1.79 \pm 0.18	2.88 \pm 0.33 ^b	3.34 \pm 0.20	3.77 \pm 0.10 ^c

^a Nine animals were included at each time point except at day 77 post-exposure to *M. bovis* at which time only seven samples were included in the analysis due to mishandling of two samples. Values are presented in units of optical density readings.

^b Significantly ($p \leq 0.05$) greater than responses at 77 days post-exposure to *M. bovis*.

^c Significantly ($p \leq 0.05$) greater than responses at 174 days post-exposure to *M. bovis*.

detected immediately following (3 days) both times of administration of PPD for CCT, days 77 and 174 post-exposure to *M. bovis*.

4. Discussion

$\gamma\delta$ T cells of mice and humans are responsive to mycobacterial antigens and are reported to have an anti-inflammatory role in acquired immunity to tuberculosis infections (Kabelitz et al., 1990; Havlir et al., 1991; O'Brien et al., 1991; Ladel et al., 1995; D'Souza et al., 1997). Although cattle have expanded populations of $\gamma\delta$ T cells (Hein and Mackay, 1991; Wyatt et al., 1994), a distinct role for $\gamma\delta$ T cells in acquired immunity to *M. bovis* infection of cattle is not described. In a study by Pollock et al., 1996, it was determined that the numbers of WC1⁺/ $\gamma\delta$ T cells within the peripheral blood of infected cattle decrease early in infection and then increase; suggesting that $\gamma\delta$ T cells traffic to developing lesions and then clonally expand. Indeed, $\gamma\delta$ T cells have been detected within DTH reaction sites of infected cattle and deer (Doherty et al., 1996; Palmer et al., 1999). Likewise, we have determined in the present study that bovine $\gamma\delta$ T cells represent a significant proportion of the T cells responding to PPD_b in the recall proliferative response of *M. bovis*-infected animals. Additionally, we determined that a large percentage of bovine $\gamma\delta$ T cells proliferated in non-stimulated PBMC cultures from either *M. bovis*-infected or non-infected cattle. The relevance of this latter finding, however, remains unclear.

Three days post-injection of PPD for skin test hypersensitivity responses, *M. bovis*-infected cattle had a significant boost in their T cell proliferative and IFN- γ response to PPD_b. Proliferative and IFN- γ responses were not detected, however, from PBMC isolated from non-infected cattle that had also received PPD for CCT. Analysis of T cell subset responses revealed that the boost in the T cell proliferative response of infected cattle was due to an increase in the numbers of CD4⁺ cells responding. Comparative cervical testing has been shown to boost *M. bovis*-specific lymphocyte blastogenic and antibody responses in BCG vaccinated red deer (*Cervus elaphus*, Griffin et al., 1993). Also, BCG-vaccinated calves have increased IFN- γ production in response to in vitro stimulation with PPD_b immediately following CCT (Buddle et al., 1995). In the same

study, non-vaccinated, infected calves did not show an increase in IFN- γ production; although, their response may have already reached a maximum detectable level (Buddle et al., 1995). A more recent study has demonstrated a boost in the in vitro proliferative but not IFN- γ responses to PPD_b and PPD_a in two of four *M. bovis*-infected cows resulting from a recent injection of PPD_b for CCT (Rhodes et al., 2000a,b). Additionally, cattle sensitized to *M. bovis* by injection of heat-killed organisms have significantly greater IFN- γ responses 3 days after caudal fold skin testing than before testing (Whipple et al., 2000). Other studies with cattle have demonstrated a decreased lymphocyte proliferative response to PPD_b 7–10 days post-intradermal injection of PPD (Doherty et al., 1995; Hall and Thoen, 1983). This “desensitization” to sequential injections with PPD is the basis for the wait period before retesting of cattle (Radunz and Lepper, 1985). Regardless, it is clear from the present findings that the lymphocyte proliferative and IFN- γ responses of *M. bovis*-infected calves to PPD can be boosted by intradermal administration of PPD for hypersensitivity testing and that CD4⁺ cells are responsible for the boosted proliferative response.

Previous reports have demonstrated that CD8⁺ cells from *M. bovis*-infected cattle respond to *M. bovis* antigens (Liebana et al., 1999; Pollock et al., 1996). In the present study, the CD8⁺ cell proliferative response of infected cattle was minimal and not statistically significant. We, however, utilized a different challenge route (e.g., environmental transmission as opposed to intranasal inoculation) and thus the cattle may have been at a different stage of infection as those previously reported by Liebana et al., 1999 and Pollock et al., 1996. Indeed, these two studies suggest that CD8⁺ cells respond late in the course of *M. bovis* infection and it is possible that CD8⁺ cells may have responded in our assay if we had obtained samples at later time points after *M. bovis* exposure. It is also possible that the choice of antigen for re-stimulation (e.g., the soluble antigen, PPD_b) may have biased the response towards a CD4⁺ and $\gamma\delta$ TCR⁺ cell proliferative response. Antigen presentation of soluble antigens is mainly via an exogenous route (e.g., phagocytosis and presentation through MHC class II) for CD4⁺ cells or direct presentation for $\gamma\delta$ TCR⁺ cells. Yet, in the study by Liebana et al., stimulation with a soluble sonicate of *M. bovis* or soluble PPD stimulated a CD8⁺ cell proliferative response. Thus, the antigen used in the present study, PPD_b, should have induced an in vitro CD8⁺ cell proliferative response if CD8⁺ cells were primed by infection.

In the present study, the B cell proliferative response of infected cattle to PPD_b was 5–10 times less than that of the response of T cells (e.g., CD3⁺ cells). It is possible that PPD, which is comprised mainly of denatured small molecular weight protein fragments, is a poor stimulant of B cell proliferation. Thus, the in vitro recall proliferative response of B cells to PPD was poor and usage of other *M. bovis* antigens in vitro may have stimulated a greater B cell proliferative response.

A pitfall of the PKH67 proliferation assay is that it is difficult to determine if proliferation of individual lymphocyte subsets is antigen specific or due to stimulation from cytokines such as IL-2 produced by other antigen-stimulated lymphocyte subsets. Detection of lymphocytes that have undergone multiple divisions, however, is suggestive of primary or antigen specific proliferation. Many of the proliferating CD3⁺, CD4⁺, and $\gamma\delta$ TCR⁺ cells were in the 6th through 9th generations, indicating an antigen specific

proliferative event (data not shown). Thus, it is likely that these subsets are, indeed, *M. bovis*-specific.

In summary, it was determined that CD4⁺ and $\gamma\delta$ TCR⁺ cells isolated from *M. bovis*-infected cattle are the predominant T cells responding in a recall proliferative response to PPD_b. The $\gamma\delta$ TCR⁺ cell proliferative response was remarkable in two aspects: $\gamma\delta$ TCR⁺ cells comprised the largest number of T cells responding to PPD and a large number of $\gamma\delta$ TCR⁺ cells proliferated in non-stimulated cultures. The CD4⁺ cell proliferative response of infected cattle to PPD was also significant. Unlike the $\gamma\delta$ TCR⁺ cell response, the CD4⁺ cell proliferative response was boosted by in vivo administration of PPD for CCT. A boost in the IFN- γ response of infected cattle was also detected post-administration of PPD for CCT. Although tempting to speculate, it is still unclear as to whether the boosted IFN- γ response is correlated with the increased proliferative capacity of *M. bovis*-specific CD4⁺ cells or not.

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